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PRINCIPAL INVESTIGATOR: Martha E. Betson, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, MA 02114-2698

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13. ABSTRACT (Maximum 200 Words) The Rho effector protein kinase N (PKN) has been implicated in prostate cancer. To study the role of PKN and closely related PRK2 in prostate cancer progression, lentiviral small hairpin RNA constructs have been obtained and which knock down expression of PKN and PRK2 in human cells. The constructs will be introduced into prostate cancer cells to study the role of PKN and PRK2 in cellular processes related to tumorigenesis. To identify novel components of the PKN signaling pathway, a genetic screen has been undertaken in the fruit fly <i>Drosophila melanogaster</i> , which has a well-conserved Pkn gene. So far two potential genetic interactors for Pkn have been identified: CKII α -i3 and RHOGAP71E. Screening is still ongoing so more interactors may be discovered. For any interactors identified, the mechanism of interaction with Pkn and the conservation of the pathway in humans will be investigated. Taken together these studies should lead to an increased understanding of a poorly characterized signaling pathway in flies and humans which may play a role in prostate cancer progression.				
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Introduction

Prostate cancer is the second leading cause of cancer death among men in the Western world. However, the molecular mechanisms underlying the development and progression of this disease are poorly understood. In an attempt to shed light on prostate cancer progression and identify novel markers I am focusing my research on the Rho effector PKN (protein kinase N). This protein kinase has recently been implicated in prostate cancer since levels of PKN protein are upregulated in prostate cancer relative to normal prostate tissue. In addition, PKN binds the androgen receptor (AR), a key protein in prostate cancer progression, and promotes AR-dependent transcription (1). The specific aims of my project are to investigate the involvement of PKN family proteins in prostate cancer, and to identify and characterize novel components in the PKN signaling pathway, which may also play a role in prostate tumorigenesis. To fulfill these aims I am using small inhibitory RNAs (siRNA) to knock down expression of PKN and the closely-related protein kinase C-related kinase 2 (PRK2) in prostate cancer cell lines, and then looking at the effects of knock down on cellular processes involved in tumorigenesis. In addition, I am using the fruit fly *Drosophila melanogaster* as a tool to identify novel proteins in the PKN signaling pathway, since the fly has a well-conserved Pkn gene (see figure 1) and many signaling pathways operating in cancer are conserved between flies and humans (2,3).

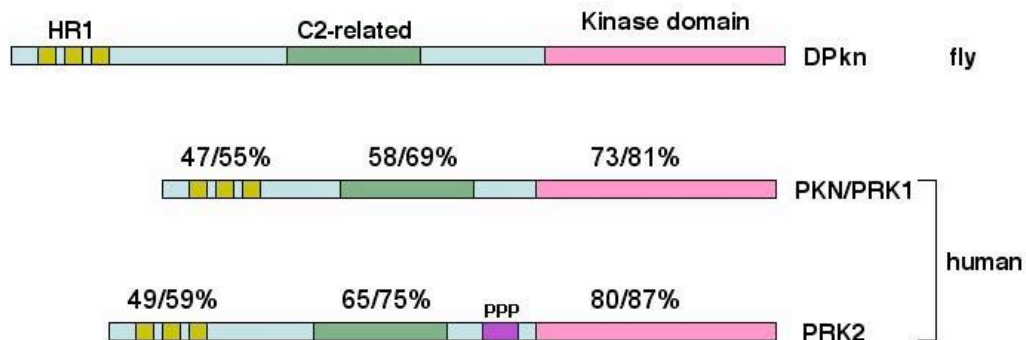


Figure 1. Domain structure of PKN family proteins. Percentage identity/similarity between fly and human proteins is shown above the domains. The HR1 domain mediates binding to Rho and Rac small GTPases. The C2-related domain shows some similarity to the C2 domain of PKCs.

Body

Task 1. To characterize the role of PKN family proteins in prostate cancer

Previous studies have indicated that PKN is expressed in prostate cancer cells (1). To confirm this and check that PRK2 is also expressed, I obtained two commercially available antibodies: a mouse anti-PKN antibody (Transduction Labs) and a rabbit anti-PRK2 antibody (Cell Signaling). Both antibodies recognized single bands of the expected sizes in PC3 cells, confirming expression of PKN and PRK2 in these cells (data not shown).

As a first attempt to knock down PKN and PRK2 expression in prostate cancer cell lines I designed 21-base-pair siRNA oligonucleotides (oligos) targeting two different regions in the human PKN transcript/mRNA sequence and two regions in the PRK2 sequence. When designing these oligos, ensured that the sequences used were not identical to any sequences in the human genome apart from PKN and PRK2. Oligos were transfected into PC3 cells and knockdown of PKN and PRK2 was assessed by western blotting. However, I failed to obtain strong and consistent knock down, despite trying a variety of different transfection conditions. It is unclear whether the problem was inefficient knock down *per se* or inefficient transfection of the oligos into the PC3 cells.

At this time the possibility arose of establishing a collaboration with the laboratory of William Hahn at the Dana Faber Cancer Institute to generate small hairpin RNAs (shRNA) against PKN and PRK2 in a lentiviral vector. The advantages of using the lentiviral system to introduce shRNAs into prostate cancer cell lines are that the efficiency of DNA transduction is much higher than when using standard transfection techniques and that stable cell lines expressing the shRNAs can be generated. In addition, once the virus has been produced in can be used to transfect multiple different prostate cancer cell lines. The Hahn lab cloned five 21-base-pair sequences from human PKN and five from human PRK2 into the lentiviral pLKO.1ps vector (see Table 1 for sequences used).

Construct	Sequence
<u>PKN</u>	
G9	cgaattccggcccagtgggga
G10	ctgctcggccaccaacctgag
G11	cccctccctccgcgcggggac
G12	tccgaattccggcccagtggg
H1	ccacctgctcggccaccaacc
<u>PRK2</u>	
C2	gccaccaatagcttctgagtt
C3	gcaggaattaaatgcacatat
C4	ccaccattttatacctaccata
C5	gcacattcatactgatgtctt
C6	gcagcagaaattggatgatat

Table 1. Sequences used to generate PKN and PRK2 shRNA constructs in pLKO.1ps

As an initial test the plasmids generated were transfected into human HEK293 cells, since these cells can be efficiently transfected. The ability to of the shRNAs to knock down PKN and PRK2 in these cells was assayed. Lysates were made from the cells, run on a gel and probed for PKN and PRK2 (see figure 2). As obvious from the blot, construct C3 specifically knocks down PRK2 and construct G12 specifically knocks down PKN whereas the other construct shows no effect.

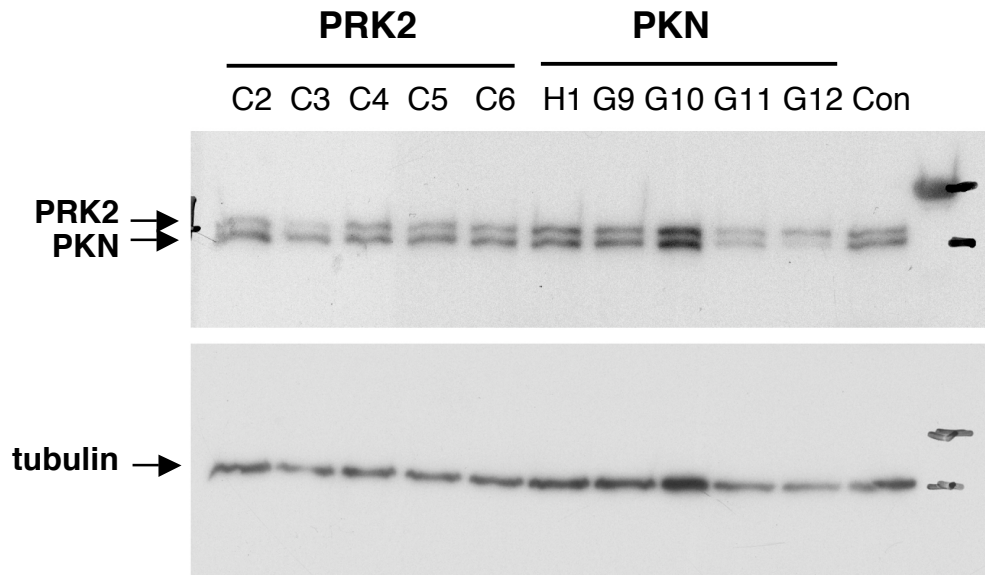


Figure 2. ShRNA-mediated knock down of PRK2 and PKN in HEK293s. Constructs C2 to C6 contained PRK2 sequences where as constructs H1 and G9 to G12 contained PKN sequences. Empty vector was used as a control (Con).

To produce lentivirus for infection of PC3 cells, the shRNA constructs were cotransfected into 293 cells with packaging (pCMV5ΔR8.91) and envelope (pHCMV-VSV-G) plasmids. After 48 hours the lentivirus-containing supernatant from the cells was used to infect PC3 cells. Unfortunately in early infection attempts, the infection efficiency was very low. I am currently optimizing the viral packaging and infection steps to improve infection efficiency. Once efficient infection is achieved, I plan to generate stable cell lines expressing the shRNAs which can knock down expression of human PKN and PRK2.

A number of different prostate cell lines will shortly be available in the lab. This will include 13 prostate carcinoma cell lines, 2 normal prostate lines and 1 benign prostate hyperplasia line. These cells will provide a useful resource for cell culture studies on PKN and PRK2. Firstly, it can be determined whether there is an upregulation of one or both proteins in a range of different prostate cancer cell lines. Secondly, the cells can be used to see whether there is any correlation between increased PKN and PRK2 expression and particular cellular characteristics including

invasiveness and androgen-independence. Such information can be used to direct future shRNA experiments on PKN and PRK2.

Task 2. To identify novel components of the Rho-Pkn signaling pathway by undertaking a genetic screen in Drosophila

I proposed to undertake a dominant modifier screen in *Drosophila* to identify novel components of the Rho-Pkn signaling pathway. In brief, this involves overexpressing a gene of interest (in this case Pkn) in a tissue (such as the wing or eye) where it produces a visible, non-lethal phenotype, crossing these flies to flies carrying different mutations and then screening in the next generation for those mutations which suppress or enhance the phenotype. The mutations which modify the phenotype are likely to occur in genes which function in the same signaling pathway as the gene of interest. This technique has been used successfully in the lab to identify novel components of signaling pathways (4, 5).

The first step was to generate a suitable phenotype for screening. The UAS/Gal4 system has been used extensively in *Drosophila* to drive expression of a gene of interest in particular tissue (6). I found that driving expression of full length Pkn in the wing or eye using this system did not produce a visible phenotype. This was unsurprising since full length mammalian PKN has been shown to exist in an inactive folded conformation. Therefore, I cloned the kinase domain of Pkn alone, which has been shown in mammalian system to be constitutively active, into the UAS vector to generate UAS-Pkn*. Expression of this domain in the eye using the Sevenless and Eyeless GAL4 produced a mild rough eye phenotype. Driving expression of the Pkn kinase domain in the wing gave phenotypes of varying severity depending on the driver used. These phenotypes included multiple wing hairs and wing blistering and shriveling and are summarized in table 2. Similar wing and eye phenotypes were observed with two different UAS-Pkn* lines. Figure 3 shows representative images of the wings and eyes overexpressing Pkn*.

Driver	Tissue	UAS-Pkn*	UAS-PKC53E*
Cy6	wing margin	missing wing material	missing wing material in some cases
32B	wing, eye	shrivelled, planar polarity defects, mwh	ectopic vein material
71B	wing	abnormal hairs	no phenotype
A9	wing	shriveled and blistered, planar polarity defects, mwh	ND
en	wing posterior	buckling, planar polarity defects, mwh in posterior half	ectopic vein material (not fully penetrant)
Act88F	wing (IFM)	no phenotype	ectopic vein material
VE	wing vein	no phenotype	no phenotype
Sev	eye	mild rough eye	no phenotype
Eyeless	eye	mild rough eye	no phenotype
GMR	eye	Rough eye	rough eye

Table 2. Phenotypes induced by overexpression of the Pkn and PKC53E kinase domain. mwh stands for multiple wing hairs; IFM stands for indirect flight muscle.

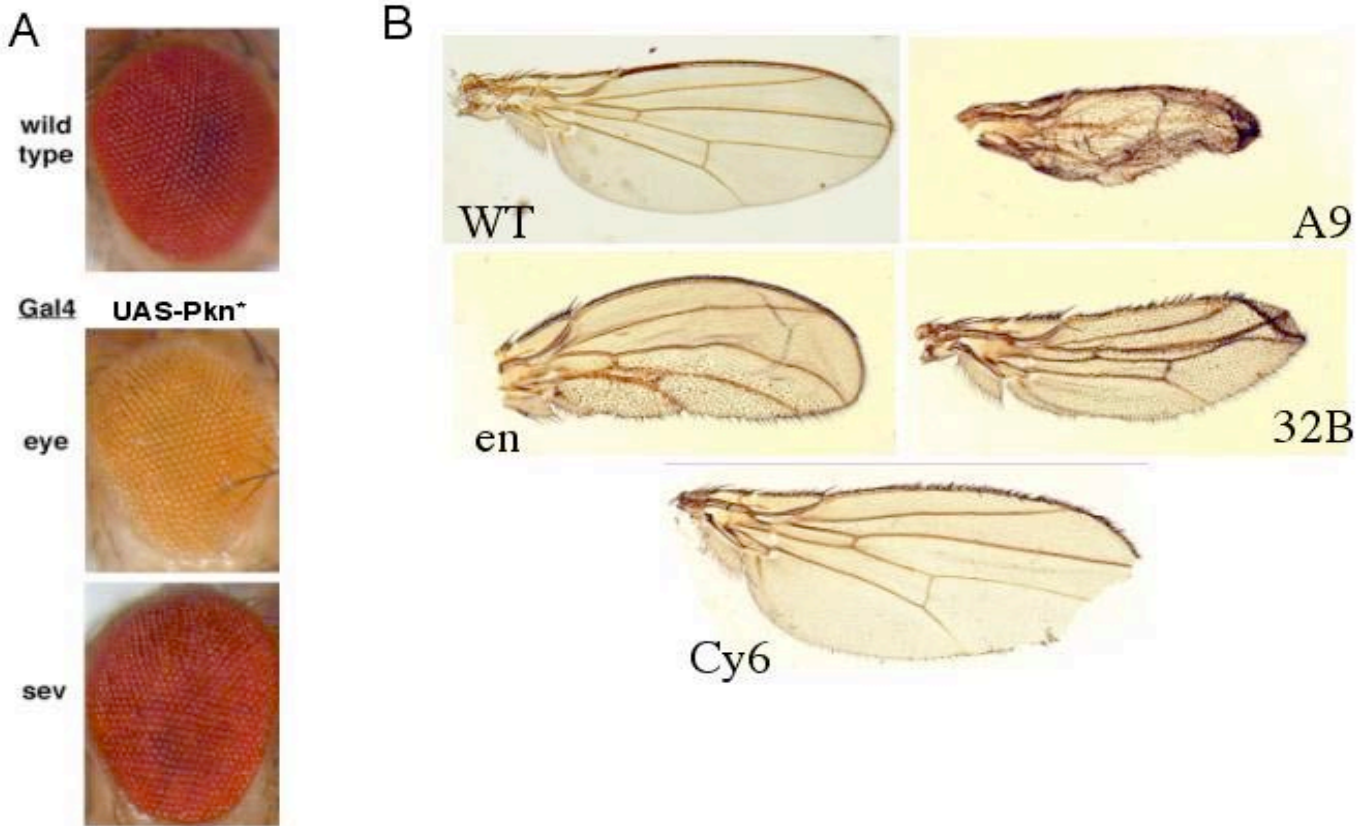


Figure 3. A) Rough eye phenotypes induced by driving expression of UAS-Pkn* in the eye. B) Phenotypes induced by driving expression of UAS-Pkn* in the wing.

To confirm that the phenotypes generated were specific to Pkn* and not purely the consequence of expressing a kinase domain in the wing and eye, the kinase domain of PKC53E was also cloned into the UAS vector. PKC53E is a classical protein kinase C and has a kinase domain closely related to that of Pkn (65 % similar). Overexpression of this in the eye produced little or no phenotype. However, in the wing it produced very different phenotypes to Pkn: in general extra wing vein material (see Table 2 and figure 4).

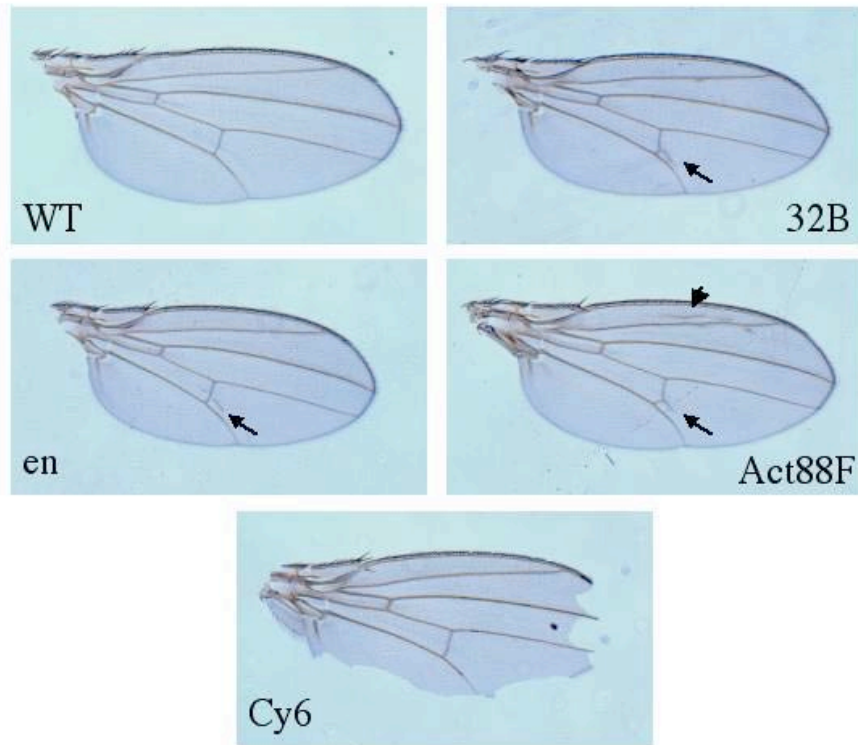


Figure 4. Phenotypes induced by driving expression of UAS-PKC53E* in the wing.

The phenotype generated using the Engrailed-Gal4 (en-GAL4) driver seemed the most consistent of the Pkn* phenotypes observed. I recombined en-Gal4 and UAS-Pkn* onto the same chromosome and made a stock. Unfortunately I discovered that this UAS/Gal4 combination caused substantial pupal lethality and so was not suitable for generating the large number of adult flies that are necessary for screening. In parallel I had also generated lines expressing the Pkn kinase domain directly under control of the GMR promoter, which drives expression of the Pkn protein in the eye. I found that one copy of GMR-Pkn* gave no phenotype but two copies in cis gave a mild rough eye which was enhanced with three copies (Figure 5). The rough eye phenotype is particularly marked at the anterior part of the eye and is stronger in females than males. Stocks for recombinants carrying three copies of GMR-Pkn* were viable and fertile and so I decided to use them for screening. Since the rough eye phenotype results from overexpression of the kinase domain of Pkn without the amino terminal regulatory region, I would expect to pull out downstream components of the Pkn signaling pathway rather than upstream components in the screen.

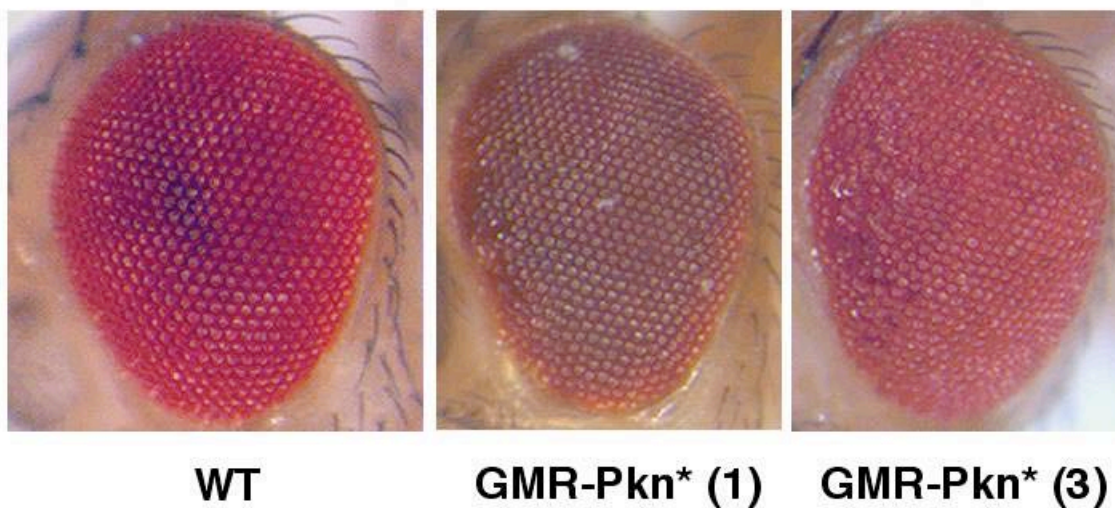


Figure 5. Rough eye phenotypes induced by expression of one copy (GMR-Pkn* (1)) or three copies (GMR-Pkn* (3)) of the Pkn kinase domain.

As a first step in screening, I crossed the GMR-Pkn* flies to each stock in the deficiency kits for the second and third chromosomes. The deficiency kits are sets of mapped deletions which span 85% of the fly genome. In the first round screen of the deficiency kit for the second chromosome, I identified 6 suppressors and 50 enhancers. 7 suppressors and 58 enhancers were identified on the third chromosome. Enhancers are less likely to be specific than suppressors. One can imagine, for example, how two different mutations in separate signaling pathways which each produce a mild rough alone, could produce an enhanced rough eye when combined. Thus I decided to focus on the suppressors rather than the enhancers.

I first retested the suppressors for modification of the Pkn* rough eye phenotype. Next I tested whether the suppressors were specific for Pkn*, rather than acting through suppression of the GMR promoter. I did this by crossing them to two other GMR lines which produce a rough eye: GMR-p21 (one copy of the p21 cDNA under control of the GMR promoter) and GMR-rok* (two copies of the Rok kinase domain under control of the GMR promoter). Four deficiencies which suppressed the Pkn rough eye phenotype failed to suppress the p21 or Rok rough eye: stock 2604 on the second chromosome, and stocks 2577, 3126 and 1931 on the third chromosome (see figures 6 and 7). These deletions also did not suppress the rough eye phenotype produced by expressing the kinase domain of PKC53E under control of the GMR promoter. Since the PKC5E kinase domain is 65% similar to that of Pkn, this strongly suggests that the suppressors are specific for Pkn. I also tested whether the four deletions I identified could suppress the wing phenotype of the UAS-Pkn*/en-Gal4 line. Unfortunately none of the suppressors were able to suppress this wing phenotype (data not shown). There are a number of possible reasons for this:

the wing phenotype may be too strong to be modified through removing only one copy of a gene; the suppressors may only participate in Pkn signaling in the eye, not in the wing; or maybe the deletions identified are not true suppressors of Pkn. Since it is difficult to distinguish between these possibilities at this stage, I decided to continue work on the suppressors I had identified.

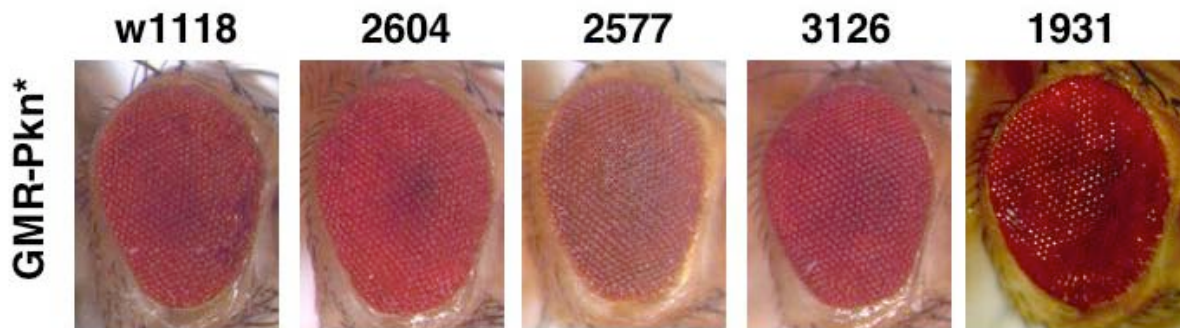


Figure 6. Suppression of the GMR-Pkn* phenotype. 2604, 2577, 3126 and 1931 represent four deficiencies which suppress the rough eye phenotype when crossed to GMR-Pkn* flies. For comparison, w1118 represents the “unsuppressed” rough eye phenotype observed when wild type flies are crossed to GMR-Pkn* flies.

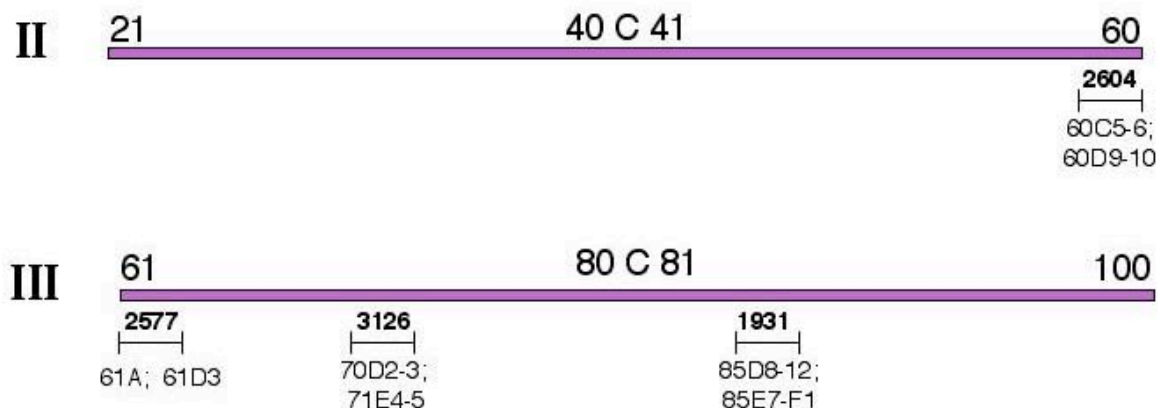


Figure 7. Diagram to show where the suppressors map. Deficiency 2604 uncovers a region at the end of the second chromosome (II). 2577, 3126 and 1931 are distributed along the third chromosome (III).

The suppressors identified in this screen are large deletions and in each case remove 60 - 140 genes from the fly genome. Thus the next step was to try and narrow down the region within

each deficiency which was responsible for the suppression of the rough eye phenotype and eventually to identify a single gene in region which could suppress. To this end I obtained a number of deletions overlapping the deficiencies identified in the screen. These came from a variety of sources: from the Bloomington stock center, from the Exelixis collection and from the Szeged stock center in Hungary. The deletions from the Bloomington center were the least useful since they tend to be large. In addition, the boundaries of the deletions are defined cytologically and therefore are not very precise. The deletions from Szeged and Exelixis are smaller and molecularly defined so it is clear exactly which genes are within the boundaries of the deletion.

Unfortunately there were no deletions available which overlapped deficiency 2604 on the second chromosome. Thus the region responsible for suppression could not be narrowed down further in this case. However there were a number of deletions overlapping the deficiencies identified on the third chromosome. These were tested and the regions responsible for suppression were narrowed down to cytological region 61C7-61C9 (for 2577), 71B1-71E1 (for 3126) and 85D24 – 85E1 (for 1931). Each of these regions contains a number of genes (see Table 3). Mutants are available in around half of these genes from stock centers, the Exelixis collection and individual labs. I am currently in the process of obtaining mutants in these genes and testing them for suppression of the Pkn phenotype. A summary of my progress so far is presented in Table 3.

Cytolocation	No. of genes	No. of genes with mutants available	No. of genes tested	Suppression?
60C5-60D10 (2604)	62	33	11	No suppressors
61C7-9 (2577)	17	8	6	1 suppressor? - CkII α -i3
71B1-71E1 (3126)	35	16	12	1 suppressor? - RhoGAP71E
85E1-85E5 (1931)	24	12	11	No suppressors

Table 3. Summary of screen to date.

As can be seen from Table 3 there are still a number of alleles to test, but two potential suppressors have been identified so far: CkII α -i3, and RhoGAP71E. It must be noted that these results are preliminary and that much more needs to be done to confirm that these genes represent bona fide genetic interactors for Pkn. CKII α -i3 stands for “CKII- α subunit interactor-3”. Very little has been reported on the function of this gene in *Drosophila*, and the two alleles tested for genetic interaction with Pkn are viable and fertile. Of these two mutants, one showed a mild suppression whereas the other did not suppress. Thus it will be important to retest the mutants for genetic interactions and test the two other available alleles.

Two out of the three RhoGAP71E alleles tested suppress the Pkn* rough eye. Limited phenotypic information is available for the RhoGAP71E gene. Mushroom body neuroblast clones for one unspecified RhoGAP71E P element allele show a reduction in cell number and an

axon guidance defect (7). In addition, of the six reported alleles one is lethal recessive but the rest are viable. I will test all six alleles for suppression of the Pkn phenotype and then check for specificity of the interaction.

In the case that the screen is unsuccessful and I fail to find specific genetic interactors for Pkn, I plan to use a biochemical approach, Tandem Affinity Purification (TAP) together with mass spectrometry, to identify novel Pkn binding partners. The TAP method is an affinity purification technique, which was developed for purification of protein complexes from yeast. Recently it has been used successfully to isolate protein complexes from *Drosophila* cultured cells (8). I have cloned the full length Pkn cDNA into pMK33-NTAP and pMK33-CTAP vectors, which will allow for expression of Pkn with an N-terminal or C-terminal TAP tag in *Drosophila* cultured cells. After performing affinity purification using TAP-tagged Pkn as a bait, any proteins which specifically associate with Pkn will be identified by mass spectrometry. Biochemical associations can be tested for physiological relevance by looking for genetic interactions between Pkn and mutants in genes encoding the proteins identified.

Key Research Accomplishments

- Lentiviral shRNA constructs generated which knock down levels of PKN and PRK2 protein when introduced into HEK293
- Transgenic flies generated carrying UAS-Pkn*, UAS-PKC53E*, GMR-Pkn* and GMR-PKC53E*
- Overexpression of the Pkn kinase domain in wing and eye using the UAS/Gal4 system or the GMR promoter shown to produce phenotypes which can be used for dominant modifier screen
- Phenotypes have been demonstrated to be specific for Pkn since overexpression of the PKC53 kinase domain produces different phenotypes
- Screen of the second and third chromosome deficiency kits with GMR-Pkn* completed
- 4 deletions identified which specifically suppress the GMR-Pkn* rough eye phenotype and do not suppress GMR-p21, GMR-rok*, or GMR-PKC53E*
- Narrowed down the regions of suppression to 60C5-60D10, 61C7-61C9, 71B1-71E1 and 85D24-85E5.
- In the process of the testing mutants in regions for suppression. Two potential suppressors identified so far: CkII α -i3 and RhoGAP71E
- Generated TAP-tagged Pkn constructs for expression in *Drosophila* tissue culture cells

Reportable Outcomes

Abstracts: “Genetic analysis of Rho effector kinase, PKN, in *Drosophila*” in Program and Abstracts for 45th Annual *Drosophila* Research Conference, Washington DC, March 24-28, 2004

Presentations: Posters at 45th Annual *Drosophila* Research Conference, Washington DC, March 24-28, 2004 and MGH Cancer Center Retreat and Scientific Advisory Board meeting, Woods Hole, October 14th-15th, 2004: “Genetic analysis of Rho effector kinase, PKN, in *Drosophila*”, and at MGH Cancer Center Retreat and Scientific Advisory Board meeting, Woods Hole, September 30th – October 1st, 2005: “Genetic studies of *Drosophila* Pkn”.

Infomatics: transgenic flies: UAS-Pkn*, GMR-Pkn*, UAS-PKC53E*, GMR-PKC53E*

Conclusions

I have generated and obtained many reagents which I can use for the study of PKN family proteins in prostate cancer cells. I have acquired antibodies which recognize PKN and PRK2 in PC3 cells, and shRNA lentiviral vectors that specifically knock down human PKN and PRK2 in human cells. I am currently optimizing the lentiviral production and infection techniques, and will soon be able to generate stable prostate cancer cell lines expressing the PKN and PRK2-shRNAs. Then I can analyze the effects of knock down of PKN and PRK2 in these cells with particular reference to cellular processes involved in tumorigenesis. This may provide novel insights into the mechanisms underlying prostate cancer development.

In terms of identifying new components of the Pkn signaling pathway, I have generated transgenic fly lines overexpressing Pkn with wing and eye phenotypes which can be used for genetic screens. I have screened the deficiency kits for the 2nd and 3rd chromosomes and identified 4 deficiencies which specifically suppress the rough eye induced by expression of Pkn*. Using smaller deletions overlapping the regions uncovered by the deficiency, I have narrowed down the regions responsible for suppression. By testing mutants in genes in this region I have identified two potential suppressors: CkII α -i3 and RhoGAP71E. The most closely related human protein to CkII α -i3 is intersectin 2. CkII α -i3 shows 21% identity and 42% similarity to intersectin 2 along most of its length, but intersectin 2 is a much larger protein and contains multiple domains including a Dbl homology domain (9). The region of homology between CkII α -i3 and intersectin 2 does not lie in any of these recognizable domains, however.

The potential interaction between Pkn and RhoGAP71E is intriguing, given that Pkn is reported to be an effector of Rho1 and that RhoGAPs (Rho GTPase activator proteins) are upstream regulators of Rho proteins. Since RhoGAPs generally function to downmodulate Rho activity it might be anticipated that mutations in a RhoGAP would enhance rather than suppress the Pkn mutant phenotype. However, the overexpressed Pkn* lacks the Rho binding domain and so is unlikely to be under Rho1 control. Perhaps a feedback loop is in operation whereby Pkn activates RhoGAP71E and thereby downmodulates Rho activity. The most closely related human protein to RhoGAP71E is ARHGAP20, which shows 32% identity and 51% similarity to RhoGAP71E along the length of, and a bit beyond, the RhoGAP domain. ARHGAP20-1 is poorly characterized but the gene is disrupted in a translocation associated with one case of B-cell chronic lymphocytic leukemia. ARHGAP20 expression is also upregulated in 22 cases of B-cell chronic lymphocytic leukemia (10).

The screening strategy I have adopted to look for novel components of the Pkn signaling pathway looks promising, since two potential genetic interactors for Pkn have already been identified, and there are still more genes to test. The challenges now will be to confirm that the genetic interactors identified represent bona fide components of the Pkn signaling pathway in *Drosophila* and to determine their mechanism of interaction with Pkn. Ultimately the goal is to determine whether the signaling pathways characterized in *Drosophila* are conserved in humans and what role they play in prostate cancer initiation and progression.

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